

REMARKS**Claim rejections - 35 U.S.C. § 112.**

Claims 83-85 were rejected under 35 U.S.C. § 112, first paragraph, because the Examiner states the specification does not reasonably provide enablement for transgenic plant expressing a recombinant animal viral antigen protein, at a level of about 0.03% or more of total soluble protein, at a level of about 0.05% or more of total soluble protein, or at a level of about 0.1 % or more of total soluble protein.

Applicants have amended claims 83-85 to recite a transgenic plant expressing a recombinant animal viral antigen protein, wherein the protein is expressed at a level of about 3-10 ng/mg of protein. Support for a transgenic plant expressing a recombinant animal viral protein at a level of about 3-10 ng/mg is found on page 29 of the specification. Additionally, support for claim 84 is also found on page 29. Claim 85 has been cancelled. Applicants respectfully request Examiner to withdraw this rejection.

Claim 98 was rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art in which it pertains or with which is most nearly connected to make and/use the invention, for reasons or records set forth in the Office Action of May 29, 2002, in which the Examiner agrees that Applicants have disclosed how to make transgenic tobacco and tomato plants that expresses a recombinant viral surface antigen protein, but asserts Applicants have not disclosed how to make transgenic plants that express a recombinant animal viral antigen protein at a level of about 0.03% or more of total soluble protein, at a level of about 0.05% or more of total soluble protein, or at a level of about 0.1% or more of total soluble protein. Further, the Examiner agrees that an antigen can elicit an immune response, but that this is dependent on dosage and the manner in

which it is administered. The Examiner finds the declaration does not show evidence of an immune response.

As stated previously, Applicants have amended claims 83-84 to recite a transgenic plant expressing a recombinant animal viral antigen protein, wherein the protein is expressed at a level of about 3-10 ng/mg of protein. Support for a transgenic plant expressing a recombinant animal viral protein at a level of about 3-10 ng/mg is found on page 29 of the specification. Additionally, support for claim 84 is also found on page 29.

Applicants are submitting herein for consideration by the Examiner the declaration of Dr. John Howard which discloses experimental support for the generation of an immune response in an animal upon exposure to transgenic plants transformed with viral.

Specifically, the declaration shows that a transgenic corn plant expressing the recombinant viral antigen led to the generation of mucosal immune response. The data shows that expression of TGEV-S antigens at high levels in corn and that these proteins delivered in the seed elicit protective responses.

Applicants have shown through the declaration previously submitted and in the present declaration, that following the procedures of the specification, a viral antigen protein was produced, fed to an animal, and a mucosal immune response observed.

Applicants are also herein submitting a publication of Wigdorovitz et al, "Induction of a Protective Antibody Response to Foot and Mouth Disease Virus in Mice Following Oral or Parenteral Immunization with Alfalfa Transgenic Plants Expressing the Viral Structural Protein VP1", *Virology* 225,347-353 (1999), to show that according to Applicants' invention the induction of a protective antibody response in animals fed transgenic plants expressing a viral antigen successfully occurred as Applicants described in their specification.

Specifically, the reference of Wigdorovitz demonstrates that mice, parenterally or orally, immunized with leaves obtained from transgenic plants, developed a similar virus-specific immune response that was able to protect the animals from experimental challenge with the virus. See the para. bridging pg. 349 col. 2 to pg. 350, col. 1 under "Induction of an immune response in orally immunized mice" and the Discussion section in general. The presence of a transgene in the plants was confirmed by PCR and their specific transcription demonstrated by RT-PCR. (See pg. 348, col. 1 under "Production and genetic analysis of transformed plants" and "Detection of transcriptional activity in the transgenic plants".)

Additionally, Applicants are herein disclosing the reference of Kapusta et al in "A plant-derived edible vaccine against hepatitis B virus," *FASEB J.* 13, 1796-1799 (1999), which shows transgenic lupin callus expressing hepatitis B virus surface antigen (HBsAg), which was fed to mice. These mice developed significant levels of HBsAg-specific antibodies. This study further demonstrates that according to Applicants' invention that antigens expressed in plants and administered orally can induce a specific antibody response in animals. (See pg. 1796, col. 1, lines 11-15; pg. 1797, col. 1, lines 11-14; pg. 1797 Material and Methods section in general).

Applicants' specification is enabling as Applicants disclose starting on page 24 how to make transgenic plants that express a recombinant animal viral antigen protein at a level of 0.03% or more of total soluble protein, at a level of about 0.05% or more of total soluble protein, or at a level of about 0.1% or more total soluble protein.

Furthermore, Applicants disclose on page 20 under Example 1, the type of vector to be used. Additionally, on page 8-9 and on page 20, under Example 1, Applicants disclose the type of animal viral protein to be expressed. On page 29, the types of plants to be transformed. With respect to the level at which recombinant protein must be expressed in order for transgenic plant

tissue to elicit an immune response against a viral antigen when the tissue is orally administered to an animal are generally well understood in the art and would not take undue experimentation. Lastly, on page 15, line 32, for example, Applicants disclose the manner in which the tissue should be administered to an animal in order to elicit an immune response. Thus, Applicants' specification is enabling.

Claim rejections 35 U.S.C. § 102

Claims 73-75, 88, 91 and 99-100 were rejected under 35 U.S.C. § 102(e) as being anticipated under Goodman et al. (U.S. Patent No. 4, 956, 282). The Examiner states the antigenic property of the expressed protein is considered to be an inherent property of the protein itself. The Examiner asserts that Goodman teaches the expression of animal viral antigen proteins in transgenic plants and that the antigenic properties of the animal viral antigen protein disclosed by Goodman are considered to be inherent to the protein.

Applicants respectfully submit that the antigen viral properties of the animal viral antigen protein disclosed by Goodman are not inherent to the proteins. [I]nherency may be relied upon where and only where the consequence of following the reference disclosure always inherently produces or results in the claimed invention. See, e.g., *W.L. Gore Associates Inc. v. Garlock Inc.*, 220 USPQ 303, 314 (Fed. Cir. 1983), *cert denied*, 469 US 851 (1984). If there is not a reasonable certainty that the claimed subject matter will not necessarily result, the rejection fails. See *In re Brink*, 164 USPQ 247 (CCPA 1970). Therefore, an Examiner who relies on the theory of inherency "must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Int. 1990).

Applicants respectfully submit that the Examiner has failed to establish by fact or technical reasoning that the antigenic property of the animal viral antigen protein disclosed by Goodman is inherent to the proteins. It is not a given among those skilled in the art that animals will always have an antigenic response to the oral administration of plant tissue per se. The Examiner has failed to recite a source for this statement and Applicants point out that plant tissue in the form of corn grain and other animal feed are orally administered to animals by farmers and other regularly, and Applicants are unaware of an antigenic response being a given accepted fact. Goodman does not provide that a viral antigen can be expressed in a plant and cause a mucosal immunogenic response. At most, Goodman is an invitation for one skilled in the art to attempt to express viruses in plants.

Although not conceding to the Examiner's rejection, Applicants have amended claims 73-75, 88, 91, and 99-100, thereby rendering this rejection moot.

Claim 73 has been amended by limiting it to a viral antigen capable of inducing a mucosal immune response. Claims 74-75 which depend from claim 73 contains by virtue of this dependency all the limitations of claim 73 and should therefore be in allowable form. Applicants respectfully request Examiner withdraw this rejection.

Applicants have amended claim 88 to recite the plant is administered orally. Dependent claim 88 contains by virtue of this dependency all the limitations of independent claim 73. It is believed that claim 88 is in allowable form. Applicants respectfully request Examiner to withdraw this rejection.

Dependent claim 91 contains by virtue of its dependency, all the limitations of independent claim 73. It is believed that claim 91 is in allowable form. Applicants respectfully request Examiner to withdraw this rejection.

With respect to claim 100, Applicants have amended this claim to read on a plant comprising a protein which triggers a mucosal immunogen response to a viral protein. Applicants respectfully request that Examiner withdraw this rejection.

Conclusion

Reconsideration and allowance is respectfully requested.

Respectfully submitted,



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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : ARNTZEN, et al.
SERIAL NO : 09/767,734
FILED : SEPTEMBER 29, 2000
TITLE : VACCINES EXPRESSED IN PLANTS

Grp./A.U. : 1638
Examiner : BUI, PHUONG T.
Conf. No. : 1914
Docket No. : P00245USD

DECLARATION OF DR. JOHN HOWARD UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Madam:

I, John Howard, hereby declare and state:

1. That I am the Chief Technical Officer of ProdiGene, Inc. the assignee of the above-identified application. Previously, I was Director of Biotechnology Research for Pioneer Hi-Bred International, Inc. for seven years, and Director of the Protein Products Division for two

CERTIFICATE OF MAILING/TRANSMISSION (37 CFR 1.8(a))

I hereby certify that this correspondence is, on the date shown below, being:

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☐ deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

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Date: 4/9/2003


LILA A.T. AKRAD

years. For nine years prior to this, I initiated and directed a biotechnology research program for crop improvement with Stauffer Chemical Company.

2. That I have a Ph.D. in Biochemistry from the University of California at Riverside. I have been involved in the science of the expression of proteins in plants for twenty years.

3. That I have read the office action dated December 9, 2002 and understand the Examiner is rejecting our claims regarding evidence of the generation of an immune response and that an animal upon exposure to transgenic plants transformed with viral antigens.

4. That in a study performed under my direction ("Delivery of subunit vaccines in maize seed", *Journal of Controlled Release* 85 (2002) 169-180), enclosed herein and described in detail hereinafter, we provided evidence in showing an antibody response in the animal fed a transgenic plant.

5. That two swine feeding trials were performed. The trial subjects were 10-12 day old seronegative piglets. The first trial was to examine serum from animals fed TGEV corn to determine they had produced neutralizing anti-TGE virus antibodies. The second study was designed to measure protection of piglets after a challenge with TGEV. In the first study, as outlined in the *Journal of Control Release*, piglets were divided into three groups. The animals were divided into three treatment groups. The control group received control corn meal; a second group was given TGEV corn meal, wherein the corn ratio was 50 grams of transgenic corn and 50 grams of wild-type corn. Piglets in the third group were maintained on normal rations throughout the course of the study. On day 29 all the animals were challenged with a 1 ml oral dose of virulent TGEV. Serum samples were analyzed for their ability to neutralize TGEV. Results of the neutralization titers are shown in Figure 4. Although neutralized antibodies were not detected in the serum of any piglets prior to virus exposure, administration of

whole virus resulted in a rapid induction of high levels of neutralized antibody in serum from piglets that had previously eaten TGEV corn. Therefore, a clear memory response leading to elevated levels of the neutralizing antibody was obtained and the animals fed transgenic corn containing recombinant TGEV-S antigens.

6. The second trial was designed to measure protection of piglets after a challenge with TGEV. Piglets were divided into five groups. One control group received control corn for 16 consecutive days; three groups were fed TGEV corn for either 4, 8, or 16 consecutive days, and one group received modified live virus vaccine as a positive control. The corn ration for each piglet was 50 grams wild-type corn or 50 grams transgenic corn (corresponding to approximately 2 mg of the S protein of TGEV). The piglets were then returned to regular water and medicated weaning rations. Piglets in the fifth group were orally vaccinated with the commercially available modified live TGEV vaccine on days 0 and 7, according to the label instructions. On day 18, all animals were orally challenged with a 2 ml dose of virulent TGEV. Following challenge, pigs were scored twice daily for signs of diarrhea and other symptoms such as dehydration and depression, anorexia, vomitus, moribund or death to give a total clinical score. To confirm viral challenge, fecal samples were collected from randomly selected animals within any group that produced watery diarrhea. These samples were tested for TGEV activity by inoculating confluent swine testicular (ST) cells in culture and staining by specific immunofluorescence.

7. That the percent morbidity incidence showed that 50% of the piglets fed wild-type corn developed TGE clinical symptoms (Fig. 5A). However, none of the piglets that received transgenic corn for 4 days exhibited symptoms.

8. That in another study performed under my direction ("Plant-based vaccines: unique advantages", *Vaccine* 19 (2001) 2742-2748), enclosed herein and described in detail hereinafter we also showed that animals fed TGEV corn could induce protection from a subsequent challenge using TGE whole virus, suggesting that feeding animals TGEV could result in generation of a protective immune response.
9. That the trial subjects were 10 day-old pathogen free piglets that were TGEV seronegative. Piglets were divided into three groups. The corn ration for one group consisted of either: (a) 100 grams of wild type corn or; (b) 50 grams of transgenic corn (corresponding to approximately 2 milligrams of the S protein of TGEV), mixed with 50 grams of wild-type corn; or (c) piglets orally vaccinated with the current commercially available modified live vaccine MLB-TGEV on day 0 and 7 of the study according to label directions. The piglets were then returned to regular water and medicated weaning rations.
10. That on day 12 all animals were orally challenged with a 2 ml dose of virulent TGEV. Prior work has shown that this dose should produce a clinically typical TGEV water diarrhea in 21 to 28 day old piglets that would persist for 7 to 10 days, but would not be lethal.
11. That following challenge, pigs were scored twice daily for signs of diarrhea and other symptoms of such as dehydration and depression, anorexia, vomitus moribund or death to give a total clinical score.
12. That to confirm viral challenge, fecal samples were collected from randomly selected animals within any group that produced watery diarrhea. The samples were checked for TGEV activity by inoculating confluent ST cells and staining by specific immuno fluorescence.
13. That the results showed all the piglets fed only wild-type corn developed TGEV clinical symptoms as shown in Figure 2A. By comparison, only 50% of those that received the

transgenic corn expressing the S protein exhibited symptoms. While 78% of piglets receiving a commercially modified live vaccine developed symptoms, indicating the transgenic corn vaccine was more effective in protecting the piglets.

14. That in a another study performed by Oragen Technologies & Veterinary Resources, Inc., on my behalf, described in detail hereinafter to determine if TGEV protein, corn-expressed vaccine can boost lactogenic immunity in animals that have previously been orally sensitized.

15. That in the study blood samples were collected at pre-breeding, 35 days prior to farrowing, day 14 and day of farrowing. Samples (serum, colostrums, and milk) were assayed for neutralizing titers using a varying antibody-constant virus assay.

16. That all animals were sensitized to TGEV and then boosted prior to farrowing. All animals were seronegative for TGEV at breeding. The animals were sensitized to TGEV by vaccination with a modified live TGEV vaccine. One group, Group G, did not receive any modified live TGEV vaccine and only received TGE transgenic corn throughout the study.

17. That the data showed that the TGEV transgenic corn was as effective as modified live TGEV in boosting the lactogenic immunity in animals that had been sensitized to TGEV. See as evidence the enclosed graph, "Sow Serum Response".

18. That the animals given only the transgenic TGEV corn were not sensitized to TGEV based on lack of neutralizing antibodies in serum (not shown in graph), colostrums, and milk of the vaccinated animals. However, the absence of a measurable response does not indicate that the sows did not respond. When challenged (data not shown), the sows did exhibit a measurable antibody response, known as memory response. This "memory response" was also shown in our study described above published in *Journal of Controlled Release* entitled "Delivery of Subunit Vaccines in Maize Seed" (See Fig. 4) where piglets did not show a primary response, but when

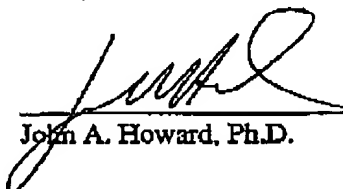
subsequently exposed to the virus, responded with a significant serum response to an extent much greater than the control group.

19. That the above-identified studies demonstrate clearly that Applicant's invention would work at the time of filing as claimed in the above-identified patent application.

20. That we have shown evidence of a generation of an immune response in an animal upon exposure to a transgenic plant transformed with a viral antigen.

21. That the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Apr 8, 2003
Date


John A. Howard, Ph.D.



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Delivery of subunit vaccines in maize seed

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Abstract

The use of recombinant gene technologies by the vaccine industry has revolutionized the way antigens are generated, and has provided safer, more effective means of protecting animals and humans against bacterial and viral pathogens. Viral and bacterial antigens for recombinant subunit vaccines have been produced in a variety of organisms. Transgenic plants are now recognized as legitimate sources for these proteins, especially in the developing area of oral vaccines, because antigens have been shown to be correctly processed in plants into forms that elicit immune responses when fed to animals or humans. Antigens expressed in maize (*Zea mays*) are particularly attractive since they can be deposited in the natural storage vessel, the corn seed, and can be conveniently delivered to any organism that consumes grain. We have previously demonstrated high level expression of the B-subunit of *Escherichia coli* heat-labile enterotoxin and the spike protein of swine transmissible gastroenteritis in corn, and have demonstrated that these antigens delivered in the seed elicit protective immune responses. Here we provide additional data to support the potency, efficacy, and stability of recombinant subunit vaccines delivered in maize seed.

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Keywords: Transgenic corn; Seed-derived vaccine; Antigen stability; Transmissible gastroenteritis virus; *Escherichia coli* heat-labile enterotoxin

1. Introduction

There are a number of elements that are critical to successful development of a vaccine (reviewed in [1–4]). A vaccine must be effective at protecting

against infection by a target pathogen without bringing harm to the host. In addition, a vaccine must be potent enough to limit dosage to a practical and affordable size. Further, a vaccine must remain potent, efficacious, and safe throughout transport and storage to the time of administration. These considerations are of high concern to the commercial animal farming industry where large numbers of animals raised in close proximity need to be immun-

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ized in a timely and cost-effective manner in order to preserve livestock health. These concerns also translate to human populations where vaccine cost, storage condition, and method of administration can limit a country's ability to effectively immunize its population, especially in developing and tropical countries. Therefore, there is need to further pursue development of new animal and human vaccines that are less expensive, more robust, and easier to deliver, yet are just as safe, potent, and efficacious as existing ones.

The use of recombinant gene technologies by the vaccine industry has revolutionized the way antigens are generated and has provided safer, more effective means of protecting host organisms against bacterial, viral, and parasite pathogens [5,6]. Recombinant subunit vaccines have become especially popular because they can be produced without using materials derived from infected animal or human hosts, thus reducing the potential for infectious contaminants. In addition, subunit vaccines can be formulated to include only essential antigens, thus limiting undesirable secondary effects associated with unnecessary components. Despite these enhancements, concerns still exist that recombinant vaccines can be cost prohibitive and, when derived from mammalian cell culture, still have the potential to be contaminated with animal pathogens. Furthermore, recombinant subunit vaccines are usually administered through parenteral injection of purified protein components thus requiring the need for controlled storage conditions to ensure stability and sterility, and equipment to administer injections.

Development of a broadly applicable oral delivery system could allow efficient widespread administration of vaccines without the need for needles, syringes, and trained personnel. This has proven to be a challenging goal since the hurdle of protein digestion must be overcome to allow effective oral delivery. Furthermore, even if an oral system could be developed where significant quantities of antigen survive the gut, there is no certainty that the antigen could be absorbed in sufficient quantities to elicit a protective immune response.

Plants are increasingly being recognized as legitimate systems for production of recombinant proteins and antigens (reviewed in [7–9]). As alternative

eukaryotic expression systems, plants have been shown to synthesize and process a variety of mammalian proteins to yield high level expression of active, properly folded proteins. Plant expression systems hold the advantage over animal cell systems in that animal viruses do not infect plants. The utility of plant systems is further supported by a growing number of studies which illustrate that plant species can be used to express foreign antigens for subunit vaccines (reviewed in [7,9,10]). When administered orally, such antigens can induce immune responses that provide protection against a subsequent challenge with a pathogen [11–13].

Maize is an established eukaryotic expression system for high-level expression and commercial production of recombinant proteins and antigens (reviewed in [14]). Recent commercialization of recombinant proteins (avidin and GUS) purified from maize seed has demonstrated the potential of this system for large-scale production of proteins that retain structural integrity and biological activity [15,16]. Protein expression as high as 0.1% of dry weight of seed has been obtained for several proteins [unpublished results]. Thus, recombinant biopharmaceutical proteins delivered in maize seed can be produced at amounts exceeding 2 kg per acre for a cost of just pennies per milligram [17]. The existing infrastructure for harvesting grain coupled with established processes for fractionation and handling of grain products provides further economical advantages to maize seed for production of valuable protein products. Furthermore, maize seed is a natural protein storage site that can be harnessed as a powerful vehicle for oral delivery of antigens. The natural bioencapsulation of proteins in maize seed may enhance antigen survival in the gut and promote antigen delivery to mucosal surfaces [11,13,18–20]. We are exploring the use of corn grain as a delivery system for edible vaccines against enterotoxigenic strains of *Escherichia coli* (ETEC) and swine transmissible gastroenteritis virus (TGEV).

Among children under 5 in developing countries, ETEC are responsible for over 650 million cases of diarrhea resulting in about 800 000 deaths each year [21]. About 20% of visitors to developing countries also contract travelers' diarrhea from ETEC [22].

The major disease agent of ETEC is the heat-labile toxin (Lt). This toxin has a multisubunit structure very similar to cholera toxin and consists of a pentamer of receptor binding (B) subunits linked to a single enzymatic (A) subunit [23]. Approximately 66% of ETEC strains harbor Lt, and in about half of these strains Lt is the only toxin present [24]. These pathogens can infect nonhuman hosts as well with enteric disease due to strains of ETEC being the most commonly occurring form of colibacillosis in pigs and calves [25]. Thus, development of an oral vaccine against ETEC can have a broad impact on animal as well as human populations.

Swine transmissible gastroenteritis (TGE) is recognized as a major cause of illness and death in piglets, particularly under conditions of intensive farming [26]. It is a highly contagious enteric disease that is characterized by vomiting, severe diarrhea and high mortality in piglets less than 2 weeks of age. The causal agent of TGE is a multisubunit, enveloped, single-stranded RNA virus, TGEV, belonging to the genus *Coronavirus* of the family Coronaviridae [26]. It contains three structural proteins designated M, N and S. The M protein is an integral membrane protein, N is a phosphoprotein that encapsulates the viral RNA genome, and S (or spike) is a large surface glycoprotein [26]. Pigs that survive a first infection are immune to subsequent infections of the virus, probably due to local mucosal immunity in the intestine through the production of S-IgA [27]. Thus, vaccines that target the activation of lymphoid tissues on the mucosal surface of the intestine are particularly attractive in the control of TGE and similar diseases. Attempts to generate a subunit vaccine that protects against TGEV using more conventional expression systems have been limited largely due to poor expression of the S protein, an important target for generation of protective (virus neutralizing) antibody, whereas plant systems have shown much promise [11,28–30].

Previously, we reported generation of transgenic maize engineered to express the Lt-B and TGEV-S antigens, and demonstrated that oral delivery of transgenic maize grain containing these antigens elicits protective immune responses in mice and piglets, respectively [11,18]. Here we report data further supporting the potency, stability, and flex-

ibility of transgenic maize seed as a delivery system for oral subunit vaccines.

2. Materials and methods

2.1. Transgenic maize lines

The development of maize lines expressing Lt-B and TGEV-S proteins in the seed has been previously reported [11,18]. Briefly, synthetic maize codon optimized versions of Lt-B (based on GenBank accession M17874) and TGEV-S (based on Miller strain of TGEV) were cloned in frame of a maize codon optimized version of the barley α -amylase signal sequence to provide a cell secretion signal at the N-terminus of Lt-B and TGEV-S for protein accumulation in the cell wall. They were placed in a maize expression cassette within a transformation vector that included right and left border sequences of an *Agrobacterium tumefaciens* Ti plasmid and the pat gene of *Streptomyces viridochromogenes* conferring resistance to the herbicide Basta. *Agrobacterium*-mediated transformation of maize embryos and selection of transformants was described [11]. Subsequent propagation of seed was performed through crossing plants derived from the original T1 seed with various maize lines, with identification of high-expressing lines performed through analysis of each subsequent generation of seed by protein-specific ELISAs. Soluble extracts were prepared as described previously [11], and soluble protein measured by the Bradford assay [31]. Quantitation of recombinant Lt-B and TGEV-S proteins was performed as described previously [11].

2.2. Fractionation of grain

Control grain as well as grain carrying the Lt-B (Lt-B corn) and the TGEV-S (TGEV corn) genes were either coarsely ground as described previously [11] or fractionated into component parts using standard milling practices. For fractionation, up to 250-lb samples of transgenic corn or control corn were cleaned using an aspirator (Kice, Wichita, KS, USA) and screens of two different mesh sizes to remove large and small impurities. Following the

cleaning, water was then added to the samples to approximately 21% moisture and the kernels were left to temper for about 2 h. The tempered kernels were cracked in a Ripple mill with an impact rotor. The cracked kernels were then dried at 38–41 °C for 40 min. Using a screen with a 0.275-cm mesh size, bran or hull (pericarp tissue), germ (embryo tissue) and large coarse grits (large pieces of endosperm tissue) were separated from medium and fine grits (small pieces of endosperm tissue) and smaller particles of meal and flour. The germ fraction was dried in the forced air oven at 38–41 °C until the moisture level was reduced to less than 12%. The recovered germ samples were flaked using a flaking roll (Ferrell-Ross, Amarillo, TX, USA) with a roll gap setting of 0.018 cm. Oil, or fat, in the germ flakes was removed by conducting repeated extractions with hexane in a stainless steel batch extractor. After solvent had been drained the samples were allowed to air dry to evaporate any residual hexane. The defatted germ samples were sifted using a 0.061-cm screen, with all material passing through the screen from sequential rounds of sifting were pooled. These samples were stored at 4 °C.

2.3. Immunization of mice

BALB/c mice were housed individually and fed a basic diet of mouse chow with water allowed ad libitum. The mouse chow was removed overnight prior to administering test samples on days 0, 7 and 21 of the study. The mice were divided into four groups, with ten individuals in each group. Test samples consisted of wild type defatted corn germ, or transgenic defatted corn germ expressing 0.33, 3.3 or 33 µg Lt-B. Blood samples were collected by conducting tail bleeds prior to the first feeding of test samples (on day 0), and on days 7, 14, 21, 28, 35 and 42 of the study. Fecal samples corresponding to material excreted over the previous 24 h were collected prior to the feeding of test samples (on day 0), and on days 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38 and 42 of the study.

2.4. Detection of anti-Lt-B antibodies in mouse serum and fecal samples

For detection of serum anti-Lt-B IgG, 96-well

plates were coated with Lt-B protein, repeatedly washed with phosphate buffered saline (PBS), and blocked with PBS containing 3% BSA for 1 h at 37 °C. The blocking solution was replaced with serum recovered from mouse blood diluted in the blocking solution. Samples were incubated for 2 h at 37 °C and then repeatedly washed with PBS containing 0.05% Tween 20 (PBS-T). For detection of anti-Lt-B IgG, anti-mouse IgG conjugated to alkaline phosphatase (diluted 1000-fold) was loaded onto plates in blocking solution for 2 h at 37 °C. Plates were repeatedly washed with PBS-T and 1 mg ml⁻¹ *p*-nitrophenylphosphate was added to each well. Following 30 min of incubation at 37 °C the absorbance at 405 nm was determined. Detection of fecal anti-Lt-B IgA was as described previously [11].

2.5. Immunization of swine

Two swine feeding trials were performed. The first trial was to examine serum from animals fed TGEV corn to determine if they had produced neutralizing anti-TGEV virus antibodies. Briefly, 10–12 day-old piglets that were TGEV sero-negative and were from a herd with a low incidence of disease were used. Twelve piglets were divided into three treatment groups; a control group receiving control corn meal, a second group receiving TGEV corn meal, and the third group receiving normal rations. On days 0–7 of the study, and again on days 15–21, all piglets in the first and second group were fasted overnight, prior to administering corn rations. Corn rations consisted of either 100 g of wild type corn or 50 g of transgenic corn (corresponding to approximately 2 mg of the S protein of TGEV) mixed with 50 g of wild type corn. The corn was mixed with medicated milk replacer to give a thick oatmeal-like consistency. Piglets in the third group were maintained on normal rations (Frostcoat, Moorman's) throughout the course of the study. Upon completion of the feeding regimen, piglets were returned to regular water and normal rations. On day 29 of the study all animals were administered a 1-ml oral dose of virulent TGEV [Purdue strain, 50% fluorescent antibody infectious dose (FAID₅₀) of 10^{7.6} FAID₅₀s per dose]. Serum samples collected on day 0, 8, 16, 23, 30 and 40, were analyzed for their ability to neutralize TGEV.

The second trial was designed to measure protec-

tion of piglets after a challenge with TGEV. Briefly, subjects were 10–12-day-old specific pathogen-free piglets that were TGEV sero-negative and were from a herd with a low incidence of disease. Fifty piglets were divided into five groups; one control group receiving control corn for 16 consecutive days, three groups fed TGEV corn for either 4, 8 or 16 consecutive days, and one group receiving modified live virus vaccine as a positive control group. The corn ration for each piglet consisted of either 50 g of wild type corn or 50 g of transgenic corn (corresponding to approximately 2 mg of the S protein of TGEV). The corn was mixed with medicated milk replacer to give a thick oatmeal-like consistency. For the four groups of piglets receiving corn, a line of prepared meal sufficient for the whole group was placed on a clean dry floor and attempts were made to ensure that each piglet received an adequate portion. The piglets were then returned to regular water and medicated weaning rations. Piglets in the fifth group were orally vaccinated with a commercially available modified live TGEV vaccine (MLV, Intervet) on days 0 and 7 of the study according to label directions. On day 18 of the study all animals were orally challenged with a 2-ml dose of virulent TGEV [Purdue strain, 50% fluorescent antibody infectious dose (FAID₅₀) of $10^{7.6}$ FAID₅₀s per dose]. This dose when administered on day 12 in a similarly designed study produced a clinically typical watery diarrhea in 21–28-day-old piglets that would persist for 7–10 days, but would not be lethal [11]. By delaying administration of virus for 6 additional days (day 18) in the study described here, it was expected that clinical symptoms would decrease in intensity slightly but would still be apparent by day 27. Following challenge, piglets were scored twice daily for signs of diarrhea (normal=0, creamy=1, watery=2) and other symptoms (dehydration and depression, or anorexia=1, vomitus=3, moribund or death=10) to give a total clinical score. Clinical symptoms scored for each study group are presented as follows: either percent morbidity incidence [(number of animals with clinical signs scoring ≥ 2 divided by total number of animals) $\times 100$], percent morbidity incidence and duration [(total number of clinical observations ≥ 2 divided by total number of pig days) $\times 100$], or clinical severity index (total clinical score divided by total number of pig days). To confirm

viral challenge, fecal samples were collected from randomly selected animals within any group that produced watery diarrhea. These samples were tested for TGEV activity by inoculating confluent swine testicular (ST) cells in culture and staining by specific immunofluorescence.

3. Results

3.1. Fractionation of transgenic maize seed

We previously reported generation of transgenic maize lines expressing recombinant Lt-B and TGEV-S antigens in corn seed [11,18]. Lt-B protein levels as high as 9% of total soluble protein have been expressed in seed, and the protein was shown to assemble into the active pentameric form in planta [18]. Expression of TGEV-S protein at levels as high as 2% of total soluble protein has been observed in transgenic corn seed, and expression correlates with the presence of a protein which is recognized by anti-TGE virus antiserum (data not shown). In the present study we started with corn seed containing Lt-B and TGEV-S proteins present at approximately 0.8 and 0.1% of total soluble protein, respectively. Corn seed is composed of several distinct compartments where proteins are known to accumulate differentially and which can be fractionated by established commercial processing methods. In order to determine whether antigens intended for oral vaccine formulation could survive the fractionation process, and to examine the distribution of these antigens within seed-derived fractions, we fractionated grain expressing the Lt-B and TGEV-S antigens using a standard milling process designed to divide the seed into component parts. The major fractions resulting from this process include germ (the plant embryo), grits (starch-rich compartment corresponding to the seed endosperm), and bran (the seed coat or 'pericarp'). A constitutive promoter was used to drive expression of Lt-B and TGEV-S gene products in these corn lines and expression is expected in all seed compartments. Analysis of proteins present in extracts derived from each of these fractions was performed using protein-specific ELISAs. Results of fractionating Lt-B corn seed are shown in Fig. 1. Lt-B protein was found in all

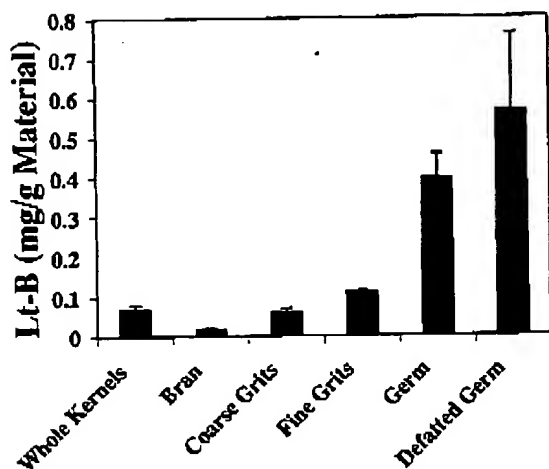


Fig. 1. Levels of Lt-B Protein in fractions of milled grain. Grain fractions were separated and analyzed for Lt-B as described in Materials and methods. Values represent mean \pm one standard deviation.

compartments of the seed. However, on a per-weight-of-tissue basis, Lt-B antigen was greatly enriched in the germ fraction. Germ is also a rich source for oil that can be extracted by hexane treatment, leaving behind a defatted germ fraction. When the Lt-B germ meal was treated with hexane to remove the fat portion of the germ, the resulting defatted corn germ meal fraction was also analyzed for Lt-B content. The results indicate that Lt-B is stable to the process of defatting the germ fraction since the measurable level of Lt-B protein was not diminished as compared to the full-fat germ fraction and actually increased, likely reflecting the increase in dry weight as a result of the loss of oil. Fractionation of TGEV corn yielded similar results (data not shown).

3.2. Stability of Lt-B and TGEV-S antigens in maize seed-derived fractions

To determine whether Lt-B expressed in maize seed and present in the germ meal fraction is stable to storage at different temperatures, aliquots (3 g) of defatted Lt-B corn germ meal were placed at either 4 or at 23 °C. At defined times, aliquots were removed and extracts were prepared. Lt-B content in extracts was analyzed by ELISA, and the results are shown in

Fig. 2A. Storage at either 4 or 23 °C for longer than a year had negligible effects on measurable Lt-B levels. The ELISA used to measure the Lt-B levels has a substantial bias toward Lt-B pentamer (the active form of Lt-B). Denaturation of an Lt-B standard from pentamer to monomer, through boiling of the protein, reduces ELISA signal by at least 20-fold without degrading the Lt-B subunits themselves (data not shown). Therefore this analysis indicates that not only does the Lt-B antigen persist

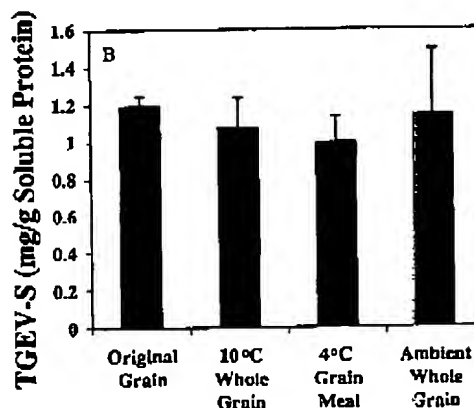
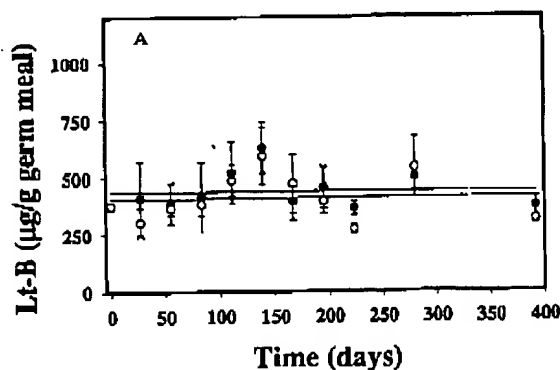


Fig. 2. Antigen stability in tissues from transgenic corn seed. (A) Measurement of Lt-B levels in defatted corn germ meal stored at either 4 °C (○) or 23 °C (●) over a 392-day period; (B) measurement of extracted TGEV-S antigen as mg antigen per g extracted soluble protein from grain or grain meal stored for 10 months at either 4 °C, 10 °C, or at ambient temperature in a grain storage facility in Iowa. Values represent mean \pm one standard deviation.

over time, but that it persists in the active, pentameric form.

Persistence of TGEV-S antigens in corn grain over time was also assessed. TGEV corn grain was harvested and stored under a variety of conditions as either whole grain or as coarsely ground whole grain meal. Grain meal was placed in storage at 4 °C. Whole grain was stored at either 10 °C in a seed storage facility where humidity was maintained at 50% (conditions routinely used to preserve seed germination potential) or at ambient temperature in a grain storage facility in Iowa where neither temperature nor humidity were controlled. Initially, TGEV corn was analyzed for TGEV-S levels in extracts prepared from a small aliquot of ground grain. After 10 months, extracts were prepared from the whole grain stored at both ambient conditions and at 10 °C as well as from grain meal stored at 4 °C. Fig. 2B shows the levels of TGEV-S antigen measured in extracts derived from samples stored at the conditions indicated. These results show that TGEV-S antigen levels persist over time either in grain meal stored at 4 °C, or in whole seed stored at higher temperatures. Importantly, persistence of the TGEV-S antigen occurred even under uncontrolled grain storage conditions indicating that temperature-controlled storage facilities are not necessary for stability of this antigen in the seed.

3.3. Potency of a corn germ-derived Lt vaccine

A previous study demonstrated that Lt-B expressed in whole corn grain and delivered orally induces both serum and secretory immune responses [11]. We further investigated the immunogenicity of Lt-B transgenic corn by examining a defatted germ fraction, in which the concentration of antigen is increased over whole kernels, and by delivering the antigen over a wider dose range, including very small doses. Mice were fed defatted Lt-B corn germ meal or defatted wild type corn germ meal, and serum and fecal samples were collected and analyzed. For all groups except the negative control (receiving wild-type corn germ meal) the serum IgG responses were evident after the first dose, or second dose for the lowest dose level, and increased throughout the study (Fig. 3A). All mice within groups fed 3.3 and 33 µg of Lt-B and 8/10 mice

within the group fed 0.33 µg of Lt-B responded with Lt-B specific serum IgG assay values exceeding 2× the average value for preimmune serum (data not shown). Thus, even 0.33 µg of the Lt-B antigen is sufficient to give a serum IgG response. This is approximately 15-fold lower than any dose previously administered in plant material [11,12]. Given the expression level of Lt-B in the defatted germ, the amount of corn material fed in this case is only 0.7 mg. Thus, clearly very small doses of Lt-B delivered in corn material can induce a response, and the amount of corn material that is administered is a very manageable dose size.

All groups except the negative control also have mucosal IgA responses (Fig. 3B). Again, a 0.33 µg dose of Lt-B antigen in defatted germ material is sufficient to induce a response. The responses are evident after the first dose and tend to cycle through the study, peaking approximately 1 week after each dose administration. The responses are greater with increasing doses of defatted Lt-B germ meal. Thus, as with a previous study using whole corn kernels, the response when using an edible plant vaccine appears at mucosal surfaces as well as in serum [11]. This indicates that edible vaccines may be particularly suited toward combating disease agents that infect or gain access to the body through mucosal surfaces.

3.4. Efficacy of a corn-derived TGEV vaccine

Previously, we demonstrated that TGEV corn, when fed to piglets, could induce partial protection from a subsequent challenge using TGE whole virus [11]. This suggested that oral administration (feeding) of TGEV corn could result in generation of a protective immune response. Therefore to test whether administration of TGEV corn leads to generation of virus neutralizing antibody, we examined serum from piglets that had been fed TGEV corn and then exposed to TGE whole virus. Piglets were fed TGEV corn for 7 consecutive days followed by normal feed for 7 days, and then finally boosted with a second 7-consecutive-day feeding of TGEV corn. On day 29 piglets were exposed to an amount of TGE whole virus that would result in a subclinical infection. Serum was collected over the experimental time course and assayed for its ability to interfere

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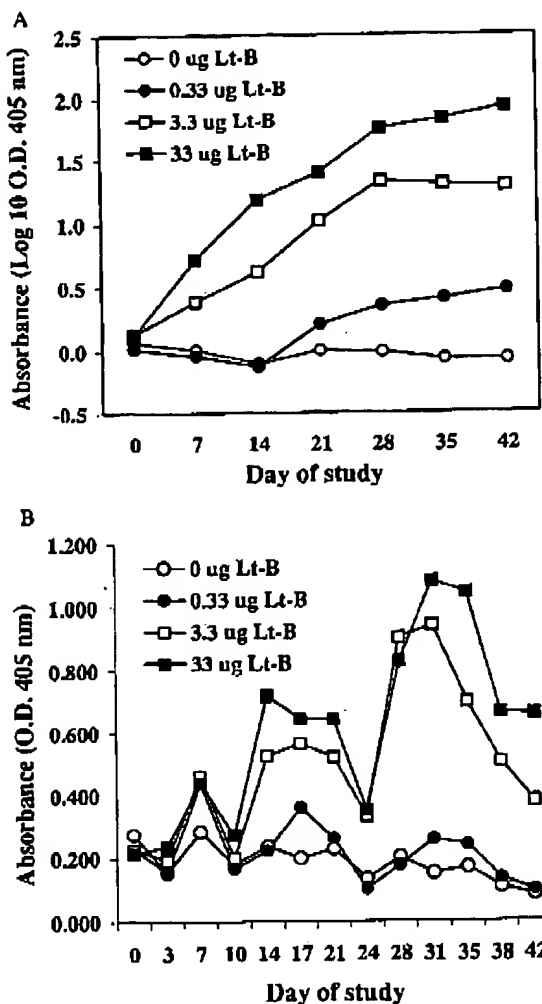
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Fig. 3. Immune responses of mice fed defatted Lt-B corn germ meal. (A) Anti-Lt-B specific IgG in serum. (B) Anti-Lt-B specific IgA in fecal material. The mean responses for the ten mice in each group are shown at defined times throughout the feeding study for animals fed defatted Lt-B corn germ meal containing the amount of Lt-B indicated.

with TGE virus infection of a swine testicular cell line in vitro. Results of analysis of the neutralization titers are shown in Fig. 4. Although neutralizing antibodies were not detected in the serum of any of the piglets prior to virus exposure, administration of whole virus resulted in rapid induction of high levels

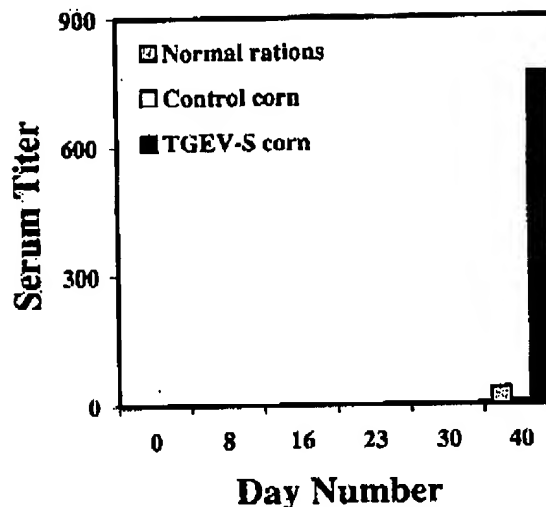


Fig. 4. Induction of TGEV neutralizing antibodies in serum from piglets fed transgenic corn seed expressing the S protein of TGEV. Mean responses are shown for the four piglets in each group that were fed normal rations, control corn, or TGEV-S corn.

of neutralizing antibody in serum from piglets that had previously eaten TGEV corn. Three out of the four animals in the TGEV corn-fed group responded with titers ranging from 512 to 2048 yielding a geometric mean titer of the four animal group of 768.5. This was in contrast to piglets that had eaten control corn meal prior to exposure to whole virus, which developed low levels of neutralizing antibody suggestive of a subclinical infection with the highest titer from any single animal in either of the control groups of only 64. Therefore, a clear memory response leading to elevated levels of neutralizing antibody was obtained in animals fed transgenic corn containing recombinant TGEV-S antigen.

Our previously reported viral challenge study employed a feeding regimen where piglets were administered TGEV corn for 10 consecutive days prior to challenge with virus on day 12 [11]. Piglets fed TGEV corn showed fewer overall symptoms of infection as compared to those fed control corn or a modified live virus vaccine, but recovery of affected piglets was slower in the TGEV corn fed group than in the whole virus administered group. Since dose amount is a critical factor contributing to the protective potential of a vaccine, we examined several

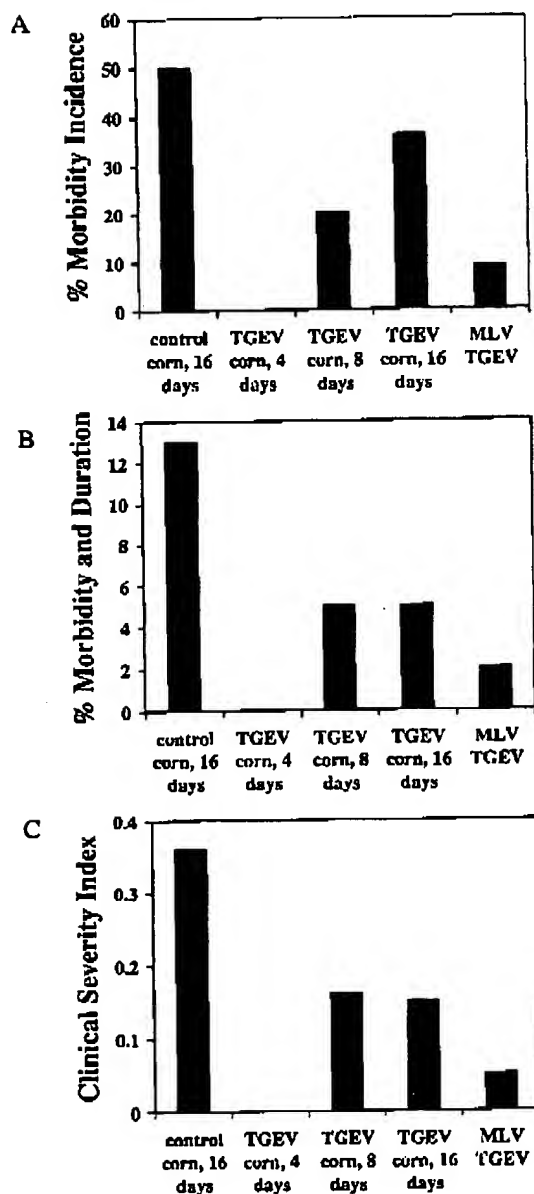


Fig. 5. Protection against TGEV of piglets fed transgenic corn expressing the S protein or a modified live vaccine (MLV). (A) Percent morbidity incidence; (B) percent morbidity incidence and duration; (C) clinical severity index. See text for definitions of clinical indices.

doses of TGEV corn for effectiveness at protecting piglets from a subsequent viral challenge. We compared the effect of feeding TGEV corn for 4, 8 or 16 consecutive days with administration of a commercial modified live TGEV vaccine for their ability to protect piglets from a viral challenge on day 18. A negative control group fed nontransgenic corn for 16 days was also included. The percent morbidity incidence shows that 50% of the piglets fed wild type corn developed TGE clinical symptoms (Fig. 5A). In contrast, none of the piglets that received transgenic corn for 4 days exhibited symptoms. Interestingly, the 8- and 16-day feedings were less effective than the 4-day feeding of TGEV corn with 20 and 36%, respectively, showing symptoms. A total of 9% of the piglets receiving the commercial modified live vaccine still developed symptoms. The percent morbidity incidence and duration as well as the clinical severity index data (Fig. 5B and C) further reflected the protection of the piglets by TGEV corn, with the greatest level of protection with a 4-day administration. Analysis (χ^2) was performed using Yates correction for continuity in calculating the statistical test, which indicated that animals fed control corn had a significantly higher rate of morbidity incidence and duration ($P < 0.001$) and severity of infection ($P < 0.001$) compared to animals that received TGEV corn for 4 days. These significant differences were comparable to the differences observed between the control corn and the modified live virus (morbidity incidence and duration ($P = 0.004$) and severity of infection ($P < 0.001$)). Taken together, these results indicate that feeding TGEV corn vaccine is effective at reducing, and under certain circumstances even eliminating, clinical disease symptoms associated with exposure to TGEV.

4. Discussion

Previously, we demonstrated expression of TGEV-S and Lt-B antigens at high levels in corn, and showed that these proteins delivered in the seed elicit protective responses [11]. Here we report data further supporting maize corn seed, and fractions thereof, as a system for delivery of potent, effective recombinant subunit vaccines that are stable, easy to deliver, and inexpensive. Two different vaccines,

derived from either whole seed or a fraction thereof, were shown to be effective at eliciting appropriate immune responses.

TGEV whole corn meal was effective at protecting piglets from a subsequent virus challenge. Our results indicate that administration of TGEV corn vaccine leads to establishment of immune memory that can be recalled as neutralizing antibodies in the serum (Fig. 4). This serum response may play a role in protection of the piglets from virus, but it is also possible that TGEV corn induces a mucosal response similar to that observed for the Lt vaccine which may also contribute to viral protection. Interestingly, a 4-day dose of the TGEV corn was shown to be more effective at preventing disease symptoms than administration of TGEV corn for either 8 or 16 days. This shows that efficacy of this oral vaccine does not require long, continuous administration of antigen and supports the case for ease of administration. In fact, in this study, extended exposure of antigen by the oral route may have limited effectiveness. However, there is no evidence that extended administration of TGEV corn had a toleragenic effect on the piglets since even in the 16-day TGEV corn group, piglets still recovered quicker, and had fewer clinical symptoms, than piglets fed control corn (Fig. 5B and C). Further, piglets fed TGEV corn for 14 days still responded with generation of significant levels of neutralizing anti-TGEV antibody when subsequently exposed to virus (Fig. 4).

Lt-B corn germ meal was also shown to be an effective source of immunogen when administered to mice, based on induction of serum anti-Lt-B IgG and fecal anti-Lt-B IgA. This is consistent with the induction of serum and mucosal immune responses observed previously using Lt whole grain [11]. Measurable serum and mucosal anti-Lt-B observed with a 0.33 μg dose of Lt-B contained in 0.7 mg corn germ meal demonstrates the utility of fractionation to enhance vaccine potency. To our knowledge, this is the lowest level of Lt-B expressed in plant material ever reported to show a measurable anti-Lt-B response. This effectiveness at eliciting a response to Lt-B is likely due to the natural bioencapsulation of plant-expressed antigens enhancing antigen survival in the gut [7,9,13,20,32]. Furthermore, it is likely that the demonstrated serum and mucosal immunogenic responses to defatted Lt-B

corn germ reported here could translate to protection of mice from a subsequent challenge with Lt holotoxin as was observed previously with the Lt whole corn vaccine [11].

The observation that antigens can survive standard grain processing and be enriched in particular fractions provides a significant degree of flexibility to vaccine formulation using transgenic corn seed without adding appreciable cost to the final product. The defatted germ fraction of Lt-B corn is highly enriched for Lt-B protein, eight-fold over whole grain. This corresponds to a significant reduction in volume of material necessary to achieve the same dose of vaccine. With an antigen level of 500 μg per gram of germ, an enormous dose of subunit vaccine could easily be incorporated into animal feed, or pelletized separately to deliver an antigen in convenient compact form, thus maximizing vaccine potency. Therefore, strategies for delivery of oral vaccines using maize seed can use existing processing/fractionation options producing a number of seed fractions that can serve as sources for antigen delivery enhancing flexibility to accommodate each subunit vaccine (Fig. 6). Here we demonstrated the effectiveness of antigen delivery in coarsely ground whole seed (TGEV vaccine) and in a germ fraction (Lt-B vaccine), but it is conceivable that grit or other fractions could also serve as sources for antigens that preferentially accumulate in the endosperm or pericarp, respectively. Furthermore, the demonstration that antigens present in either whole grain or in

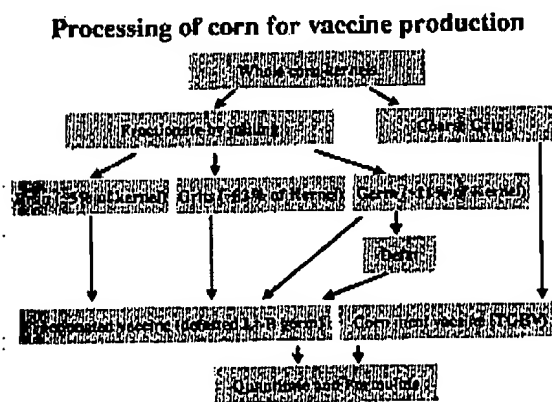


Fig. 6. Processing of corn seed for vaccine production.

the germ fraction are stable without refrigeration illustrates that vaccines produced in this fashion are amenable to low-cost transport and storage conditions.

Although the maize system has shown quite promising as a vaccine candidate that could protect against strains of ETEC which harbor the Lt holotoxin, only approximately 66% of ETEC strains harbor Lt, and in only half of these strains Lt is the only toxin present [24]. Therefore an effective anti-diarrheal vaccine suitable for broad protection of a population would likely require targeting multiple pathogens. The maize expression system is flexible enough to address such a concern. Multivalent vaccines could be designed by using transgenic lines in which multiple antigens have been co-expressed within the same seed, or formulated using corn materials from separate lines blended to include multiple antigens.

This study further illustrates the potential of corn-based edible vaccines for veterinary applications. Here we show effective protection of a common animal species, raised for human consumption. It is likely that this approach will find broad application in a number of economically significant animal species. We have preliminary data where chickens were fed Lt-B corn meal and responded with induction of anti-Lt-B antibodies (unpublished results) indicating that this technology may have application in avian systems as well. Of course this technology need not be limited to veterinary vaccine delivery. Recent human vaccine clinical trials have demonstrated that plant-based delivery systems have the potential to impact human health [33,34]. Plants systems are also potentially amenable to delivery of other proteinacious biopharmaceuticals such as antibodies, growth factors, and other medically relevant agents. The strength of the maize system for production of large quantities of stable, potent, and efficacious recombinant subunit vaccines that can be administered easily without the need for specialized equipment will likely impact both animal and human health care.

5. Conclusion

We demonstrate that seed from transgenic corn is

an effective system for oral delivery of potent, stable, and inexpensive recombinant subunit vaccines. The spike protein of TGEV and the Lt-B subunit of heat labile enterotoxin were expressed in corn seed to serve as sources of antigen for candidate vaccines to protect against two pathogenic agents, ETEC and TGEV. Lt-B antigen enriched in the germ fraction of corn seed by standard grain milling processes was shown to be potent for eliciting both serum and mucosal immune responses when fed to mice, and remained stable for at least a year when stored at either 4 or 23 °C. TGEV-S antigen in whole corn seed was shown to be stable for at least 10 months at 4 °C, 10 °C, or at ambient grain storage conditions in Iowa, and effective when fed to piglets at eliciting neutralizing anti-TGEV antibodies in the serum and protecting against a subsequent viral challenge.

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Plant-based vaccines: unique advantages

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Abstract

Numerous studies have shown that viral epitopes and subunits of bacterial toxins can be expressed and correctly processed in transgenic plants. The recombinant proteins induce immune responses and have several benefits over current vaccine technologies, including increased safety, economy, stability, versatility and efficacy. Antigens expressed in corn are particularly advantageous since the seed can be produced in vast quantities and shipped over long distances at ambient temperature, potentially allowing global vaccination. We have expressed the B-subunit of *Escherichia coli* heat-labile enterotoxin and the spike protein of swine transmissible gastroenteritis virus at high levels in corn, and demonstrate that these antigens delivered in the seed elicit protective immune responses. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Plant vaccines; Transmissible gastroenteritis virus (TGEV); *Escherichia coli* heat-labile enterotoxin (LT)

1. Introduction

Following recent advances in molecular biology, there is a growing potential for new classes of vaccines. The dissection of pathogens into their various components allows the development of specific subunit vaccines that are just as efficacious but are safer than whole pathogen vaccines. However, despite recent advances in vaccine research, the most common route of vaccination remains that of parenteral injection. The development of a broadly applicable oral delivery system remains a goal for the efficient widespread administration of vaccines, but unfortunately this has proven impractical in most cases to date. The use of subunit vaccines for oral delivery has been generally resisted because of the obvious likelihood of protein degradation in the gut. Furthermore, even if the protein were to survive within an oral delivery system, there is no certainty that trafficking the protein to the gut would be sufficient to mount an immune response.

Recently, transgenic plants have been investigated as an alternative means to produce and deliver vaccines. There are several reports demonstrating that antigens derived from various pathogens can be synthesized at high levels and in their authentic forms in plants [1–3]. When administered orally, by feeding, such antigens can induce an immune response [4,5] and, in some cases at least, result in protection against a subsequent challenge with the pathogen [6,7]. Certain antigens expressed in plants have shown sufficient promise to warrant human clinical trials [8,9]. This has led to optimism that the inherent advantages of plants can be used to dramatically change the way in which we deliver vaccines, and indeed that plants can become the delivery vehicle of choice for future vaccines. Combining the normal use of plants as human foods and as animal feed, with the production of vaccine subunit components in plant tissues, should allow vaccines to be produced at a fraction of the cost of other approaches. For the same cost, the recipient may take 100 or even 1000 times the dose of antigen that would be delivered by the parenteral injection route. We are exploring the use of corn grain as a particularly convenient delivery system for edible vaccines using both human and commercial animal examples, including

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vaccines against enterotoxigenic strains of *Escherichia coli* (ETEC) and swine transmissible gastroenteritis virus (TGEV).

Among children under five in developing countries, ETEC are responsible for over 650 million cases of diarrhea resulting in about 800 000 deaths each year [10]. About 20% of visitors to developing countries also get travelers' diarrhea from ETEC [11]. A major disease agent of ETEC is the heat-labile toxin (LT). This toxin has a multi-subunit structure very similar to cholera toxin and consists of a pentamer of receptor binding (B) subunits and a single enzymatic (A) subunit [12]. Approximately 66% of ETEC strains harbor LT, and in about half of these strains LT is the only toxin present [13]. An oral vaccine against ETEC is under development and consists of an inactivated whole cell *E. coli* component, together with an LT-B subunit component [14]. We have expressed LT-B in corn and demonstrate its immunogenicity and efficacy when fed to mice.

Swine transmissible gastroenteritis (TGE) is recognized as a major sickness and cause of death in piglets, particularly under conditions of intensive farming [15]. It is a highly contagious enteric disease that is characterized by vomiting, severe diarrhea and high mortality in piglets less than 2 weeks of age. The causal agent of TGE is a multi-subunit, enveloped, single-stranded RNA virus, TGEV, belonging to the genus *Coronavirus* of the family *Coronaviridae* [15]. It contains three structural proteins designated M, N and S. Protein M is an integral membrane protein, N is a phosphoprotein that encapsulates the viral RNA genome, and S (spike) is a large surface glycoprotein [15]. Pigs that survive a first infection are immune to subsequent infections of the virus [16], probably due to local mucosal immunity in the intestine through the activation of S-IgA. Thus, vaccines that target the activation of the mucosal surface of the intestine are particularly attractive in the control of TGE and similar diseases. We have expressed the S protein of TGEV in corn and show that it is protective in a piglet feeding trial.

2. Materials and methods

2.1. Construction of plasmids

A synthetic version of a gene encoding a variant of LT-B (GenBank accession M17874) was synthesized in which codon usage was optimized for highly expressed maize genes. A series of overlapping complementary oligonucleotides was annealed and the resulting sequence was amplified by the polymerase chain reaction. A maize codon optimized version of the barley α -amylase signal sequence was also incorporated to provide a cell secretion signal at the N-terminus of LT-B. This signal sequence is cleaved upon protein export and

results in high levels of protein accumulation in the cell wall [17]. A maize codon optimized version of the S protein of TGEV was constructed in a similar manner and also fused to the barley α -amylase signal sequence. The synthetic genes were sub-cloned into a maize expression cassette within a transformation vector that included right and left border sequences of an *Agrobacterium tumefaciens* Ti plasmid and the *pat* gene of *Streptomyces viridichromogenes* conferring resistance to the herbicide Basta.

2.2. *Agrobacterium*-mediated maize transformation

The plant expression constructs were introduced into maize using an *A. tumefaciens* mediated approach [18]. Ears of the maize line Hi-II were harvested at 9–12 days after pollination, surface sterilized in 50% bleach with Tween-20 for 30 min and rinsed twice with sterile water. Immature zygotic embryos were isolated from the ears under sterile conditions and washed twice with co-cultivation medium. A suspension of *A. tumefaciens* was added directly to the embryos and the mixture was vortexed vigorously for 30 s and incubated at room temperature for 5 min. Embryos were placed scutellum side up onto co-cultivation medium and were incubated at 19°C in the dark for 3–5 days. They were then transferred to antibiotic-containing medium without herbicide selection and were incubated for a further 3 days in the dark at 27–28°C. Subsequently, embryos and calli were transferred to fresh herbicide containing selection medium every 2 weeks, and the callus was finally transferred onto regeneration medium. Mature somatic embryos were placed in the light and allowed to germinate. Ten plants from each transformation event were transplanted to soil and were grown in a greenhouse to generate T₁ seed.

2.3. Preparation of soluble protein extracts from corn seed

Dry seeds were pulverized using a mortar and pestle and were then shaken vigorously in a tube with a steel ball bearing in the presence of approximately 500 μ l of PBST per seed. Extracts containing soluble protein were recovered by centrifugation of the homogenized tissue and collection of the supernatant. Protein concentrations were determined using an assay for protein-dye binding [19].

2.4. Quantification of recombinant LT-B in transgenic corn

A sandwich-ELISA was deployed. Between each step the 96-well assay plates were repeatedly washed with PBST. Plates were coated with 133 ng ml⁻¹ of LT-B antibody in 0.05 M carbonate/bicarbonate buffer at pH

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9.6 by incubating for several hours at 4°C. The wells were then blocked by incubating with 3% BSA in PBST at 37°C for 1 h. Soluble protein extracts were added to the wells and incubated at 4°C for several hours. Next, 50 ng ml⁻¹ of biotinylated LT-B antibody in 1% BSA/PBST was added to the wells and incubated at 37°C for 1 h. Streptavidin-alkaline phosphatase in PBST/1% BSA was then added to the wells and incubated at 37°C for 30 min. Finally, *para*-nitrophenyl-phosphate was added to each well and incubated at 37°C for 30 min, and the absorbance at 405 nm was recorded. A dilution series of a recombinant LT-B standard and non-transgenic corn were included in the assay.

2.5. Quantification of recombinant TGEV S protein in transgenic corn

A sandwich-ELISA was deployed. Between each step the 96-well assay plates were repeatedly washed with PBST. Plates were coated with antibody raised against feline infectious peritonitis virus (FIPV) which recognizes TGEV (VMRD, Inc., Pullman, WA) in 0.05 M carbonate/bicarbonate buffer at pH 9.6 by incubating for several hours at 4°C. The wells were then blocked by incubating with 5% milk/PBST at 37°C for 1 h. Soluble protein extracts were added to the wells and incubated at 4°C for several hours. Next, 387.5 ng ml⁻¹ of biotinylated FIPV antibody in 5% milk/TPBST was added to the wells and incubated at 37°C for 1.5 h. Streptavidin-alkaline phosphatase in PBST/1% BSA was then added to the wells and incubated at 37°C for 1 h. Finally, *para*-nitrophenyl-phosphate was added to each well and incubated at 37°C for 30 min, and the absorbance at 405 nm was recorded. A dilution series of a TGEV standard and non-transgenic corn were included in the assay.

2.6. Production of transgenic grain

Transgenic lines expressing high levels of recombinant protein in T₁ seed were backcrossed to commercial maize lines. Pollen from the transgenic lines was used to pollinate the commercial lines in order to bulk up transgenic seed rapidly.

2.7. Immunization of mice

BALB/c mice were housed individually and fed a basic diet of mouse chow with water allowed ad libitum. The mouse chow was removed overnight prior to administering test samples on days 0, 7 and 21 of the study. The mice were divided into five groups, with seven individuals in each group. Test samples consisted of either mouse chow with or without 50 µg pure recombinant LT-B, wild type corn, or transgenic corn

expressing 5 or 50 µg LT-B. All the mice consumed each dose in full. Blood samples were collected by conducting tail bleeds prior to the first feeding of test samples and on days 6, 13, 20 and 27 of the study. Fecal samples corresponding to material excreted over the previous 24 h were collected prior to the first feeding of test samples and on days 4, 7, 11, 14, 18, 21, 25 and 28 of the study. For the protection assay, two groups of mice were fed either wild type corn or transgenic corn expressing 50 µg LtB on the same feeding program as above. The patent mouse assay was conducted 34 days after initial feeding.

2.8. Detection of anti-LT-B Ig in mouse serum

96-well plates were coated with LT-B pentamer, repeatedly washed with PBS and blocked with 3% BSA for 1 h at 37°C. The blocking solution was replaced with serum recovered from mouse blood and diluted 11-fold in the blocking solution. Samples were incubated for 2 h at 37°C and then repeatedly washed with PBST. Anti-mouse Ig polyvalent conjugated to alkaline phosphatase and diluted 1000-fold in blocking solution was added to each well and incubated for 2 h at 37°C. Plates were repeatedly washed with PBST and 1 mg ml⁻¹ *para*-nitrophenyl-phosphate was added to each well. Following 30 min of incubation at 37°C the absorbance at 405 nm was determined.

2.9. Detection of anti-LT-B IgA in mouse fecal samples

Between each step the 96-well assay plates were repeatedly washed with PBS. Plates were coated with the ganglioside G_{M1} at 4°C for several hours and blocked with 3% BSA for 30 min at 37°C. A 1 µg ml⁻¹ solution of LT-B in PBS was added to each well and the plates were incubated at 37°C for 1 h. Lyophilized fecal pellets were resuspended in 1% BSA/PBST, incubated for 30 min and spun to remove solid matter. Samples of fecal supernatants were added to each well and incubated at 37°C for 1 h. A 1000-fold dilution of goat anti-mouse IgA was added to each well and incubated at 37°C for 1 h. Next, a 5000-fold dilution of anti-goat alkaline phosphatase conjugate was added to each well and incubated at 37°C for 1 h. Finally, *para*-nitrophenyl-phosphate was added to each well, the plates were incubated at 37°C for 30 min and the absorbance at 405 nm was determined.

2.10. LT protection assay

The patent mouse assay used here was a modification of the sealed adult mouse assay [20]. Following completion of the feeding regime, mice were fasted but allowed

water ad libitum for 24 h. For this period they were housed in cages with raised wire meshes to prevent coprophagy. Each study group was split and the mice were administered by gavage either 20 µg LT in PBS buffer or just PBS buffer. They were left for 4 h, sacrificed and dissected to assess gut swelling. The upper intestine and carcass weights were recorded, and the ratio calculated.

2.11. Swine feeding trial

The trial subjects were 10 day-old specific pathogen free piglets that were TGEV sero-negative and were from a herd with a low incidence of disease. On days 0–10 of the study all piglets in each of three groups of 10 animals were withheld from feed overnight, prior to administering corn to two of the study groups. The corn ration for a piglet consisted of either 100 g of wild type corn or 50 g of transgenic corn (corresponding to approximately 2 mg of the S protein of TGEV) mixed with 50 g of wild type corn. The corn was mixed with medicated milk replacer to give a thick oatmeal-like consistency. For the two groups of piglets receiving corn, a line of prepared meal sufficient for the whole group was placed on a clean dry floor and attempts were made to ensure that each piglet received an adequate portion. The piglets were then returned to regular water and medicated weaning rations. Piglets in the third group were orally vaccinated with the current commercially available modified live vaccine MLV TGEV on days 0 and 7 of the study according to label directions. On day 12 of the study all animals were orally challenged with a 2 ml dose of virulent TGEV (Purdue strain, titer $10^{7.6}$ FAID₅₀'s per dose). Prior work had shown that this dose should produce a clinically typical TGE watery diarrhea in 21–28 day old piglets that would persist for 7–10 days, but would not be lethal. Following challenge, piglets were scored twice daily for signs of diarrhea (normal = 0, creamy = 1, watery = 2) and other symptoms (dehydration and depression, or anorexia = 1, vomitus = 3, moribund or death = 10) to give a total clinical score. The clinical symptom data for each study group are presented as a percent morbidity incidence (number of animals with clinical signs > 2 divided by total number of animals), a percent morbidity incidence and duration (total number of clinical observations > 2 divided by total number of pig days), and a clinical severity index (total clinical score divided by total number of pig days). To confirm viral challenge, fecal samples were collected from randomly selected animals within any group that produced watery diarrhea. These samples were checked for TGEV activity by inoculating confluent ST cells and staining by specific immunofluorescence.

3. Results

3.1. LT-B corn fed to mice induces an immune response that combats LT holotoxin

We first investigated whether LT-B produced in corn would induce an immune response when fed to mice. Mice were fed ground transgenic corn seed and serum and fecal samples were analyzed for immune responses. Notably, equivalent amounts of pure LT-B or transgenic corn expressed LT-B induce similar anti-LT-B specific Ig responses in serum Fig. 1A. The response is clearly evident at 13 days after the first feeding and remains elevated for the course of the study. Doses of 5 µg of LT-B expressed in corn are sufficient to give a strong Ig response in serum, demonstrating that corn is an effective oral delivery vehicle for LT-B.

As a guide to mucosal immunogenicity, anti-LT-B specific IgA levels were recorded in fecal material of mice that had been fed LT-B expressed in corn. Responses are evident after 7 days and clearly cycle with peak responses about 1 week after each dose Fig. 1B. As with the serum Ig response, doses of 5 µg of LT-B expressed in corn are sufficient to induce a strong mucosal IgA response. Strikingly, LT-B expressed in corn induces a much greater anti-LT-B specific mucosal IgA response than pure LT-B.

In order to assess the efficacy of LT-B expressed in corn, we examined whether it could prevent gut swelling in mice exposed to the LT holotoxin. The upper intestines of a control group of mice swell up when gavaged with LT, whereas those of mice fed LT-B expressed in corn do not swell Fig. 1C. Thus, LT-B expressed in corn appears to be protective against LT.

3.2. The S protein of TGEV expressed in corn is protective against the virus

We then progressed to developing an edible vaccine against an economically important animal disease, TGE in swine. We conducted a study to compare transgenic corn expressing the S protein of TGEV with a commercial modified live TGEV vaccine. A negative control group fed wild type corn was also included. The percent morbidity incidence shows that all the piglets fed only wild type corn developed TGE clinical symptoms Fig. 2A. By comparison, only 50% of those that received the transgenic corn expressing the S protein exhibited symptoms. Interestingly, 78% of the piglets receiving the commercial modified live vaccine developed symptoms, indicating that the edible transgenic corn vaccine is more effective. By contrast, the percent morbidity incidence and duration, and the clinical severity index indicate that piglets that received the modified live vaccine appear to recover quicker than those that were fed transgenic corn Fig. 2B and C.

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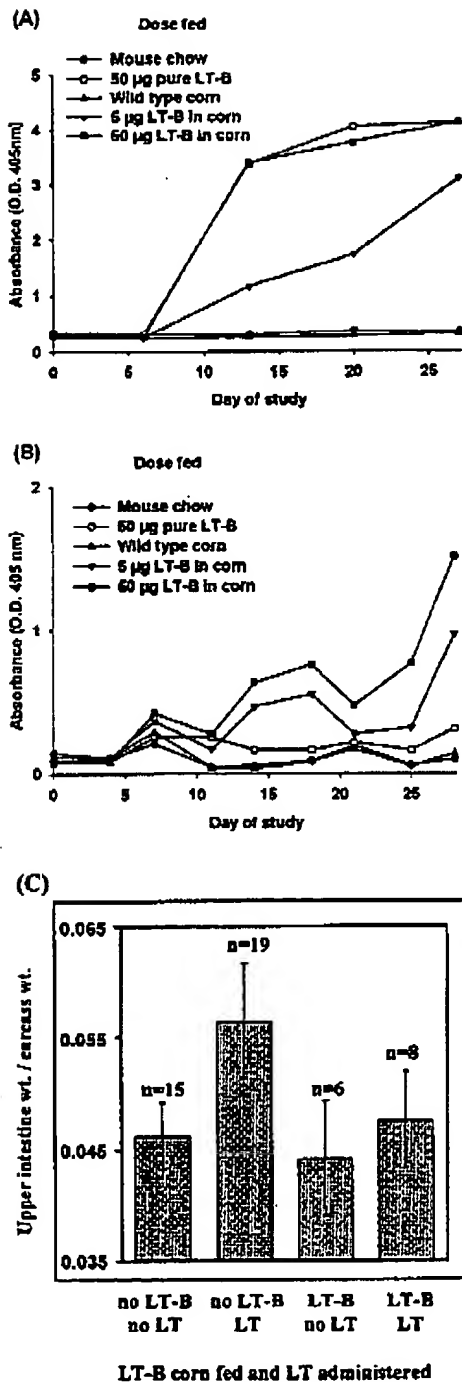


Fig. 1. Protective immune responses of mice fed transgenic LT-B corn. (A) Anti-LT-B specific Ig in serum. The mean response for the seven mice in each group is shown; (B) Anti-LT-B specific IgA in fecal material. The mean response for the seven mice in each group is shown; (C) The degree of gut swelling following challenge with LT holotoxin. Mean values for the weight ratios are shown with 95% confidence levels, and the sample size is given (n).

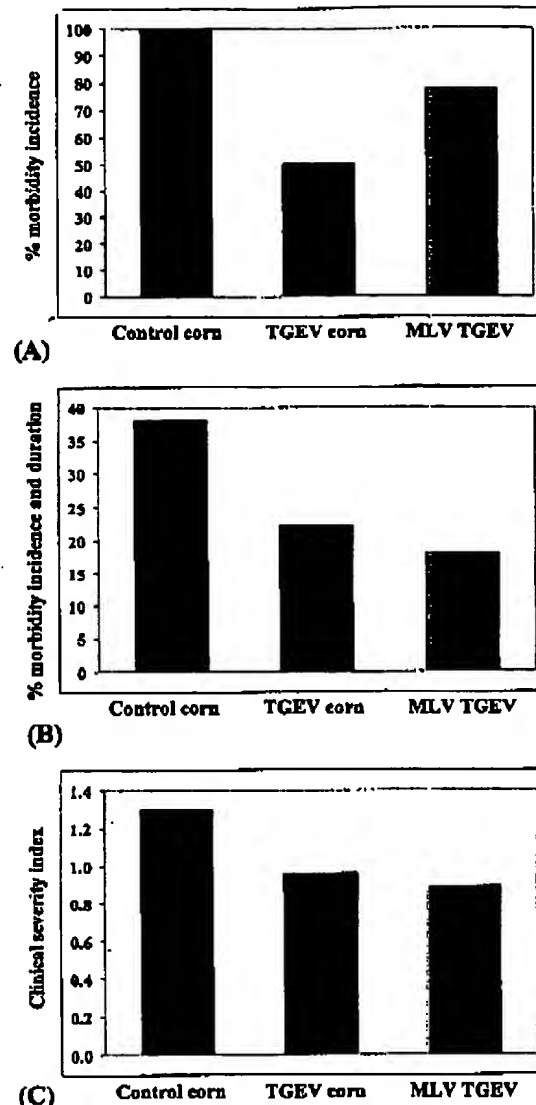


Fig. 2. Protection against TGEV of piglets fed transgenic corn expressing the S protein or modified live vaccine (MLV) TGEV. (A) Percent morbidity incidence; (B) Percent morbidity incidence and duration; (C) Clinical severity index. See text for definitions of clinical indices.

However, differences between these two groups in the percent morbidity incidence and duration, and in the clinical severity index are slight, whereas both groups are clearly protected against TGEV compared to piglets fed wild type corn. Fecal samples from piglets showing watery diarrhea were shown to harbor TGEV (data not shown).

4. Discussion

The use of transgenic plants, and in particular cereals, for vaccine production has several potential benefits

over traditional methods. Firstly, transgenic plants are usually engineered to express only a small antigenic portion of a pathogen or toxin, eliminating the possibility of infection or innate toxicity and reducing the potential for adverse reaction. Secondly, since there are no known human or animal pathogens that are able to infect plants, concerns with viral or prion contamination are eliminated. Thirdly, the successful synthesis of foreign proteins in transgenic crops rely on the same established technologies to sow, harvest, store, transport and process plant material as those currently used for food crops, making transgenic plants a very economical means of large-scale vaccine production. Fourthly, the expression of foreign proteins in the natural storage tissues of plants maximizes protein stability, so reducing the need for refrigeration and keeping transportation and storage costs low [17]. Fifthly, the formulation of multi-component vaccines is possible by blending the seed of transgenic lines expressing different proteins. Sixthly, direct oral administration is possible when the chosen proteins are expressed in commonly consumed food plants to give edible vaccines.

Orally administered vaccines are particularly efficient at stimulating local mucosal immune responses at the intestinal surface, and the integrated nature of the mucosal immune system allows other mucosal sites to also be primed [21]. The mucosal immune system is distinct and independent of the humoral immune system and is not effectively stimulated by parenteral administration of candidate antigens. Rather, the mucosal immune system requires antigen presentation directly to a mucosal surface [22]. Since mucosal surfaces form the first defense against transmissible diseases entering the body through oral, respiratory, urinary and genital routes, edible plant vaccines are an attractive means of protection against a wide range of pathogens. They could potentially be used alone or in combination with other vaccination routes.

To our knowledge the study reported here is the first example of an animal used in conventional food husbandry acquiring protection from a major disease through ingesting an edible vaccine. Moreover, by using corn as the delivery vehicle for the antigen, we utilized a conventional feed material of the animal. The protection observed in this study includes aspects of general health and vigor, reduced clinical symptoms and other factors known as criteria for disease protection. The mechanism of protection is unknown but may be an active immune response by the animal, competitive inhibition of viral receptor sites leading to non-establishment of viral infection, or interference with the viral replication process.

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Table 9. Summary of Treatment and Boosting Schedule for Induction of Lactogeneic Immunity Study.

Group	Number of pigs	Treatment	Vaccine Boost
A	8	MLV Oral - day -115 Oral - day -102 IM - day -88	TGEV Corn - Oral Days -35 to -29 Days -14 to -8 (14 days)
B	7	MLV Oral - day -115 Oral - day -102 IM - day -88	TGEV Corn - Oral Days -35 to -33 Days -14 to -12 (6 days)
C	7	MLV Oral - day -115 Oral - day -102 IM - day -88	TGEV Corn - Oral Day -35 Day -14 (2 days)
D	5	MLV Oral - day -115 Oral - day -102 IM - day -88	Control Corn - Oral Days -14 to -8 (7 days)
E	5	MLV Oral - day -115 Oral - day -102 IM - day -88	MLV - IM Day -35 Day -14 (2 days)
F	7	MLV Oral - day -115 Oral - day -102 IM - day -88	TGEV Corn - Oral Days -14 to -8 (7 days)
G	8	TGEV corn - Oral Days -115 to -113 Days -102 to -100 Days -88 to -86 Days -35 to -33 Days -14 to -12	TGEV Corn - Oral Days -35 to -33 Days -14 to -12 (6 days)

